ACID LABILE SUGAR IN PEPSINOGEN

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This note is to report that the pepsinogen molecule of swine stomach contains covalently bound sugar. In contrast, no sugar could be detected in pepsin prepared from the same pepsinogen percursor, or from twice crystallized pepsin. The covalently bound sugar in pepsinogen could be released by acid treatment, under the same conditions in which pepsinogen is converted to pepsin. The major sugar component of pepsinogen was identified as D-glucose.

Materials and Methods

All pepsin and pepsinogen preparations used in this study were of swine stomach origin. They were commercial preparations of Worthington. Crystalline pepsinogen, lots PG GJA and PG 9AA, was prepared according to Herriott (1938), and chromatographically purified pepsinogen, lots PG 8GA and PGC 7JA, according to Rajogapolan et al. (1966). The pepsin used was twice crystallized, lot PM 712.

Pepsin was also prepared from Worthington pepsinogen as follows: the solution containing pepsinogen was acidified with 0.3 N HCl to result in pH 1.7, and kept at this pH for 10 min at room temperature. After 10 min the protein was precipitated by adding a saturated solution of $MgSO_4$ to yield 60% W/W saturation. After centrifugation the precipitate was redissolved in 0.1M sodium acetate buffer pH 4.7, and dialyzed for two hr against two liters of water at 5^0.

The concentrations of pepsin and pepsinogen in the reaction mixtures used were established by their absorption at 278 nm according to Arnon et al. (1963). The amount of sugar in the proteins (0.1 μmole) was determined by the phenol-sulphuric acid method (Dubois et al. (1956)).

For the separation and identification of the sugars, two-dimensional high-voltage paper electrophoresis and descending paper chromatography were used.

In the first dimension, samples of 0.1 μmole protein per spot were applied on Whatman No. 3 MM paper and subjected to high-voltage paper electrophoresis either at pH 6.5, 60 volts per cm for 60 min, or at pH 1.9, 30 volts per cm for 2 hr. After the first run a 3 cm wide vertical strip was cut from the electrophoretogram and horizontally stitched onto a new paper (No. 3 MM) and then subjected to descending chromatographic separation in a direction perpendicular to the first run. The solvent systems used were: I, ethyl acetate-pyridine-water (2:1:2) (Isherwood and Jermyn (1951)) or II, n-butanol-acetic acid-water (4:1:5). The chromatograms were stained to reveal the position of peptides with ninhydrin reagent (2.5 gr ninhydrin, 478 ml acetone, 20 ml water and 2 ml pyridine). The reducing sugars were revealed by a silver nitrate reagent (Sharon and Jeanloz (1960)) and identified by their migrations relative to appropriate sugar standards which were treated in the same way as the samples. The major sugar component, D-glucose, was also identified by glucose oxidase spray (Salton (1960); Martinsson (1966)).

Tryptic or papain digestion of pepsinogen was performed in 0.1 M sodium phosphate buffer pH 8.0. The reaction mixture contained 1 μmole per ml pepsinogen and 400 μg trypsin or papain respectively. The tryptic or papain digest was analyzed after 24 hours incubation at 37^0.

Results and Discussion

Purified preparations of pepsinogen and pepsin were analyzed for the presence of sugar. The phenol-sulphuric acid test showed that pepsinogen from four different batches contained approximately 3 molar equivalents of neutral sugar based on a glucose standard. On the other hand, pepsin which was prepared from the same pepsinogen preparations and commercial crystalline pepsin gave a negative phenol sulfuric acid test. These results imply that during the conversion of the zymogen to the active enzyme, sugar is released from the protein.

In order to find out if the sugar present in pepsinogen is free or bound, samples of pepsinogen (0.1 μ mole) were subjected to high-voltage electrophoresis (pH 6.5) followed by chromatography in system I. Release of reducing sugar could not be detected by the silver nitrate reagent. Conversely, the treatment of pepsinogen with HCl (pH 1.7) under conditions in which pepsinogen is activated, brought about the release of free sugar as demonstrated by silver nitrate after the same electrophoretic and chromatographic separations (Fig. 1). The results obtained in the above experiments indicate that the sugar is covalently bound to the pepsinogen molecule

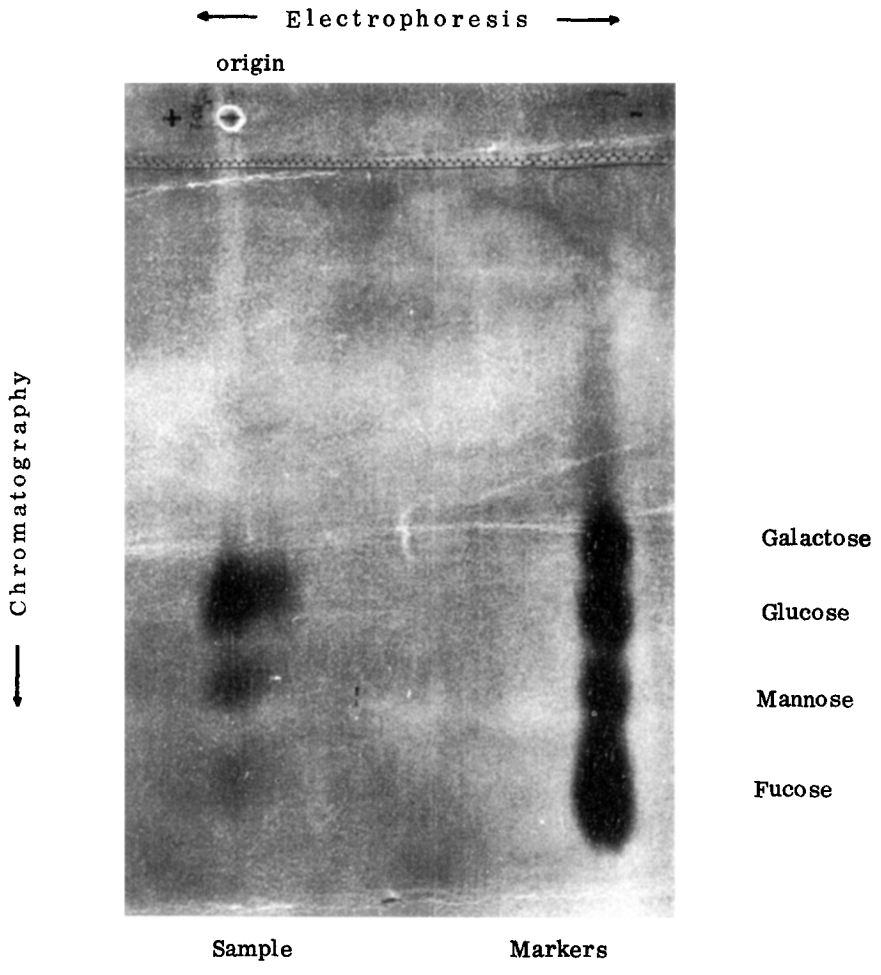

Fig. 1. The release of sugar after acid treatment of pepsinogen. An aliquot of pepsinogen, 0.1 μmole was treated with HCl, and transferred to Whatman No. 3 MM paper. Electrophoretic separation was performed at pH 1.9, 30 volts per cm for two hr in the first dimension. A vertical strip from the first dimension was sewn horizontally onto a new sheet of paper and subjected to descending chromatographic separation in solvent system I. The sugar was revealed by silver nitrate reagent.

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and is partly released as free reducing sugar after treatment with acid (see Fig. 1).

To confirm the covalent type of binding of the sugar molecule to the protein, pepsinogen was digested either by trypsin or by papain. The peptides resulting from tryptic digestion were separated by high-voltage paper electrophoresis at pH 6.5. A 3 cm wide vertical strip from the electrophoretogram was developed with ninhydrin reagent to locate the peptides. Another 3 cm vertical strip was developed with silver

nitrate reagent for the detection of reducing sugars. No reducing sugar was observed. However, when a similar vertical strip from the above electrophoretogram was horizontally stitched onto a new paper sheet (Whatman No. 3 MM) and subjected to descending paper chromatographic separation in the acidic solvent system II, at least one strong silver nitrate positive spot appeared below the positively charged peptides (see Fig. 2). This finding clearly demonstrates that in the first dimension the sugar moiety migrated together with one of the positively charged peptides, and was released only after treatment with acid. However, reducing sugar did not appear when the

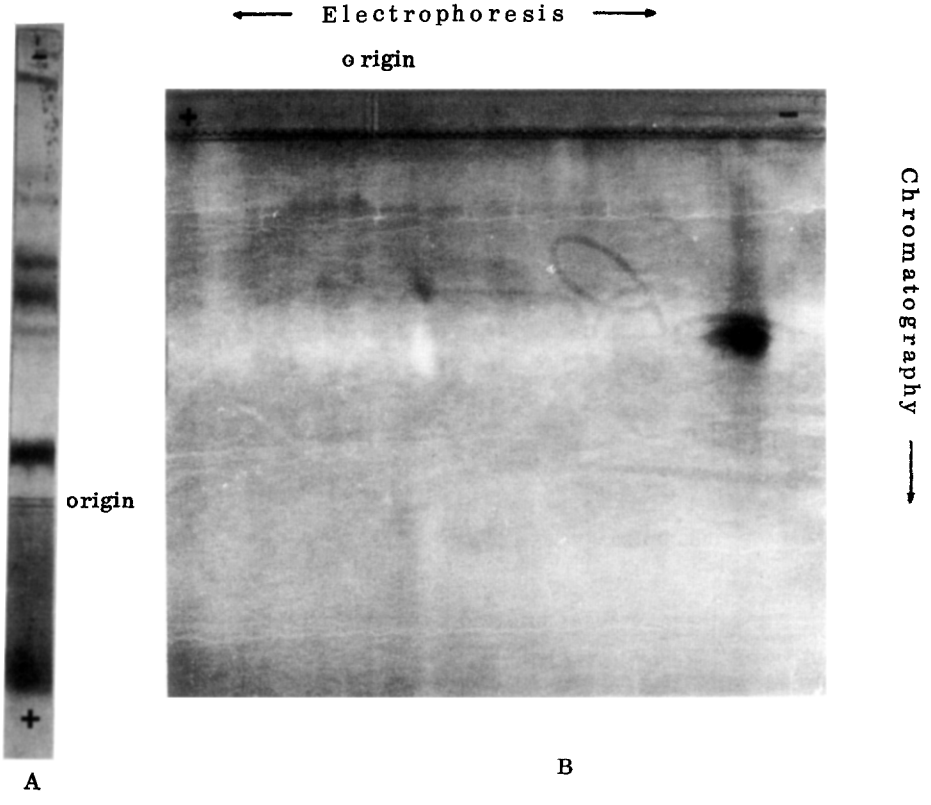

Fig. 2. The release of sugar after tryptic digestion of pepsinogen. Aliquots of pepsinogen (0.1 μmole per spot) were transferred after tryptic digestion to Whatman No. 3 MM paper. Electrophoretic separation was performed at pH 6.5. 60 volts per cm for 40 minutes in the first dimension. A. A vertical strip from the electrophoretogram was revealed with ninhydrin reagent. B. A vertical strip from the first dimension was stitched horizontally to a new sheet of paper and subjected to descending chromatographic separation in system II. Chromatogram stained with silver nitrate reagent.

chromatogram was performed in solvent system I instead of II. This indicates that the sugar molecule, even in the peptides, is bound through an acid labile bond. The same treatment was applied to locate the sugar containing peptides after papain digestion of pepsinogen. The results obtained were essentially the same as in the case of tryptic digestion, except that the glycopeptides had different electrophoretic mobilities at pH 6.5. The amino acid composition of the sugar containing peptides and the nature of the sugar-peptide bond are at present under investigation.

For the identification of the sugar moieties in pepsinogen, aliquots of the acid-treated pepsinogen were subjected to electrophoresis (at pH 1.9 or pH 6.5, followed in the second dimension by chromatographic separation using both systems). D-glucose was found to be the major sugar released and its identification was supported by a positive glucose oxidase test. The additional components were found in small amounts and might be mannose and fucose (Fig. 1).

In spite of our present lack of knowledge of the place where the sugar is bound to the pepsinogen, we wish to present, in conclusion, some speculation on the role of sugar in this protein. It is pertinent to note that the release of the sugar occurs under the same mild acid treatment (pH 1.7, 10 min, room temperature) as pepsinogen is converted to pepsin. Thic correlation may be coincidental. Preliminary kinetic experiments indicate, however, that the release of sugar precedes the release of peptides from pepsinogen, at pH 1.9. This suggests that the release of sugar by acid is an early step in the activation of pepsinogen. The presence of sugar in pepsinogen may have a function, therefore, of stabilizing the zymogen in a conformation where the active site is protected.

References

Arnon, R., and Perlmann, G.E., J.Biol.Chem. 238, 653 (1963).
Dubois, M., Giles, K.A., Hamilton, J.K., Rebers, P.A., and Smith, F., Anal. Chem. 28, 350 (1956).
Herriott, R.M., J.Gen.Physiol. 21, 501 (1938).
Isherwood, F.A., and Jermyn, M.A., Biochem. J. 48, 515 (1951).
Martinsson, A., J.Chromatog. 24, 487 (1966).
Rajocopolan, T.G., Moore, S., and Stein, W.H., J.Biol.Chem. 241, 4940 (1966).
Salton, M.R.J., Nature, 186, 966 (1960).
Sharon, N., and Jeanloz, R.W., J.Biol.Chem. 235, 1 (1960).